Comparative thermal denaturation of *Thermus aquaticus* and *Escherichia coli* type 1 DNA polymerases

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Thermal denaturations of the type 1 DNA polymerases from *Thermus aquaticus* (*Taq* polymerase) and *Escherichia coli* (Pol 1) have been examined using differential scanning calorimetry and CD spectroscopy. The full-length proteins are single-polypeptide chains comprising a polymerase domain, a proofreading domain (inactive in *Taq*) and a 5′ nuclease domain. Removal of the 5′ nuclease domains produces the 'large fragment' domains of Pol 1 and *Taq*, termed Klenow and Klentaq respectively. Although the high temperature stability of *Taq* polymerase is well known, its thermal denaturation has never been directly examined previously. Thermal denaturations of both species of polymerase are irreversible, precluding rigorous thermodynamic analysis. However, the comparative melting behaviour of the polymerases yields information regarding domain structure, domain interactions and also the similarities and differences in the stabilizing forces for the two species of polymerase. In differential scanning calorimetry, Klenow and Klentaq denature as single peaks, with a melting temperature T_m of 37 and 100 °C respectively at pH 9.5. Both full-length polymerases are found to be comprised of two thermodynamic unfolding domains with the 5′ nuclease domains of each melting separately. The 5′ nuclease domain of *Taq* denatures as a separate peak, 10 °C before the Klentaq domain. Melting of the 5′ nuclease domain of Pol 1 overlaps with the Klenow fragment. Presence of the 5′ nuclease domain stabilizes the large fragment in Pol 1, but destabilizes it in *Taq*. Both Klentaq and Klenow denaturations have a very similar dependence on pH and methanol, indicating similarities in the hydrophobic forces and protonation effects stabilizing the proteins. Melting monitored by CD yields slightly lower T_m values, but almost identical van't Hoff enthalpy ΔH values, consistent with two-state unfolding followed by an irreversible kinetic step. Analysis of the denaturation scan rate dependences with Arrhenius formalism estimates a kinetic barrier to irreversible denaturation for Klentaq that is significantly higher than that for Klenow.

Key words: CD, differential scanning calorimetry, protein folding, *Taq* polymerase, thermophilic.

INTRODUCTION

Full-length DNA polymerase 1 from *Thermus aquaticus* (*Taq* polymerase) is both structurally and functionally homologous with DNA polymerase 1 from *Escherichia coli* (Pol 1) [1–5], yet *Taq* is known to retain at least partial activity at temperatures up to 97.5 °C [1,2]. Owing to its uses in PCR and DNA sequencing, *Taq* polymerase is one of the most widely used biotechnological reagents in the world. However, the molecular basis for the thermal stability of *Taq* is understood only partially [3–5], and the thermodynamic basis for its stability has never been examined.

Both *Taq* and *E. coli* Pol 1 full-length polymerases are single polypeptide chains comprising a polymerase domain, a 3′ exonuclease (or proofreading) domain and a 5′ nuclease domain. The proofreading domain of *Taq* is inactive [2]. Figure 1 shows the domain structure of full-length *Taq*. Removal of the 5′ nuclease domain from the full-length polymerase produces the 'large fragment' domains of *E. coli* Pol 1 and *Taq*, termed Klenow and Klentaq respectively [6,7]. Both Klenow and Klentaq are functional DNA polymerases on their own. Crystal structures of Klenow and Klentaq polymerases have been determined and they show a high degree of structural similarity [4,6]. Currently, there is no crystal structure for full-length *E. coli* Pol 1.

Elucidation of both the thermodynamic and structural origins of the stability of thermophilic proteins continues to be separate but overlapping areas of active research and debate [8–13]. The current consensus on the structural origins of hyperthermal stability is that there is no consensus: each protein is unique and, whereas increased ionic interactions are the non-covalent feature correlated most prevalently with thermophily, any particular thermophilic protein may use a combination of multiple molecular strategies [8,9]. As with many thermophilic proteins, the structural rearrangements in *Taq* which are believed to be associated with high temperature stability are relatively modest. The structural comparisons between *Taq*/Klentaq and Klenow from two different laboratories focus on different aspects of the two structures. Steitz and co-workers [3] indicate that the structures reveal a small number of additional internal hydrogen bonds in *Taq* relative to Klenow and also an exchange of two ionic interactions for two hydrophobic interactions in the polymerase domain. Waksman and co-workers [4] report a decrease in the number of unfavourable electrostatic interactions in the thermophilic protein, a 'global reorganization of the charge distribution' in the proteins leading to a higher net electrostatic stabilization in Klentaq and approx. 1–1.6 % increase in the fraction of buried surface area which is hydrophobic in Klentaq relative to Klenow.

To investigate further the nature of high temperature stability of *Taq*, we directly compared the thermal melting behaviour of *Taq*/Klentaq and *E. coli* Pol 1/Klenow. The thermal stabilities of *Taq* and other thermophilic DNA polymerases have been examined previously by monitoring the loss of enzymic activity as a function of incubation time at increased temperatures. Such measurements

Abbreviations used: DSC, differential scanning calorimetry; DTT, dithiothreitol; Pol 1, DNA polymerase 1 from *Escherichia coli*; Klenow, the 'large fragment' domain of *E. coli* Pol 1; *Taq* polymerase, full-length DNA polymerase 1 from *Thermus aquaticus*; Klentaq, the 'large fragment' domain of *Taq* T_m, melting temperature.

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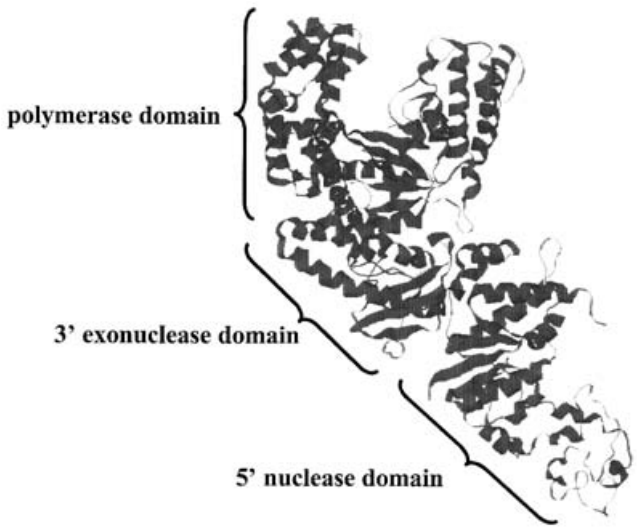

Figure 1 Structure of *Taq* DNA polymerase

The three-dimensional structure of the enzyme as determined by Steitz and co-workers [3] is shown with the structural/functional domain arrangement of the enzyme indicated (Protein Data Bank accession no. 1TAQ).

provide a useful and reproducible way of comparing relative enzymic stabilities. In the present study, however, we have collected differential scanning calorimetric (DSC) denaturation data for *Taq* and Pol 1 DNA polymerases and their 'large fragments' (Klentaq and Klenow) in an attempt to understand further the basis for the enhanced temperature stability of *Taq*. In addition, Klenow and Klentaq have been examined as a function of pH, methanol and scan rate, and their denaturation has also been monitored by CD spectroscopy. Because both polymerases denature irreversibly, thermodynamic analysis of the data is essentially precluded. As more proteins are being examined by calorimetric and thermal denaturation methods, it is becoming clear that 'thermal unfolding of most mesophilic and thermophilic proteins is irreversible' [10]. Although irreversible denaturations cannot be analysed thermodynamically, it does not mean they are devoid of information. Despite their irreversibility, the results of the present study reveal information on the domain structure, domain–domain interactions, similarities in stabilizing forces and differences in the kinetic stabilization of these two species of polymerase. The results presented here are the first of their kind for either polymerase.

EXPERIMENTAL

Proteins were expressed and purified as described previously [14,15]. No surfactants were used during preparation, storage or calorimetry of the proteins. Calorimetric experiments were conducted within 1–2 weeks after protein purification. For each DSC experiment, proteins were dialysed against a buffer containing 10 mM K_2PO_4/10 mM EDTA/2 mM dithiothreitol (DTT) at the pH values indicated for each experiment. One of the challenges in DSC is to find solution conditions under which the protein of interest denatures reproducibly without aggregating or precipitating. EDTA treatment and removal of all salt from the denaturation buffer were necessary to prevent precipitation of the polymerases after heating. EDTA treatment also eliminated irregular low-temperature heat-capacity anomalies, presumably by removing excess bivalent cations from the polymerases. Values of pH < 9.5 also caused precipitation of full-length *Taq* polymerase. Protein concentrations were determined using the Bradford assay [16]. Varying the protein concentrations from 0.2 to 3.0 mg/ml did not significantly alter any calorimetric value. Experiments were performed using both a MicroCal VP-DSC and a MicroCal MC-2 with a scan rate of 1 °C/min, except where indicated for the determination of the scan rate dependence of Klentaq and Klenow. Subtraction of the buffer and transition baselines yield the excess heat-capacity curves, which were normalized for the protein concentration and analysed using the MicroCal Origin DSC software, version 5.0. Reversibility was examined by rescanning both after full denaturation and after heating to the melting temperature T_m and then cooling.

Scan rate dependence was determined using the methods described by Sanchez-Ruiz et al. [17]. The scan-rate-dependent shift in T_m for denaturation was fitted to the equation:

$$\frac{\text{scan rate}}{{T_m}^2} = \frac{AR}{E_a} e^{-E_a/RT_m}$$

such that a plot of $\ln(\text{scan rate}/{T_m}^2)$ against $1/T_m$ yields a slope $-E_a/R$, where E_a is the activation energy for denaturation, R the gas constant and A the pre-exponential factor in the Arrhenius equation.

Thermal denaturations were also performed with optical monitoring by CD on an AVIV Model 202 CD spectrometer. Spectra were recorded from 226 to 216 nm in 1 nm steps at each temperature. Protein concentration was 0.05 mg/ml in 10 mM potassium phosphate buffer (pH 9.5). For Klenow, data were collected from 5 to 71 °C in 1–3 °C intervals, with the smallest temperature steps (1 °C) in the transition region. For Klentaq, data were collected from 60 to 110 °C in 1–2 °C intervals, again with the smallest steps (1 °C) in the transition region. Protein was incubated for 5 min with stirring at each new temperature in a rectangular cuvette (1 cm path length) with a screw top seal. CD signals at 219, 220, 221 and 222 nm were used for analysis of the unfolding curves. Thermal denaturation curves were fitted to a modified form of the van't Hoff equation, which simultaneously fits the native and denatured baselines and the transition region to obtain the T_m and ΔH values for denaturation [18]:

$$\Delta\varepsilon = (m_n T + b_n) + (m_d T + b_d)\left(\frac{K}{1+K}\right)$$

where

$$K = \exp[-\Delta H(1 - T/T_m)/RT]$$

Here, m_n, m_d and b_n, b_d are the slopes and intercepts of the native- and denatured-state baselines respectively and T is the temperature. Data were fitted using the program KaleidaGraph (Synergy Software, Reading, PA, U.S.A.).

RESULTS

We have examined the thermal denaturation of type 1 DNA polymerases from *T. aquaticus* (*Taq*) and *E. coli* (Pol 1). Figure 2 shows characteristic DSC scans for *Taq* and Pol 1 polymerases and their Klentaq and Klenow 'large fragments', after subtraction of the buffer baselines. The area under a DSC curve normally yields the calorimetric enthalpy of denaturation of the protein, ΔH_{cal}. The position of the peak yields the T_m for denaturation. A

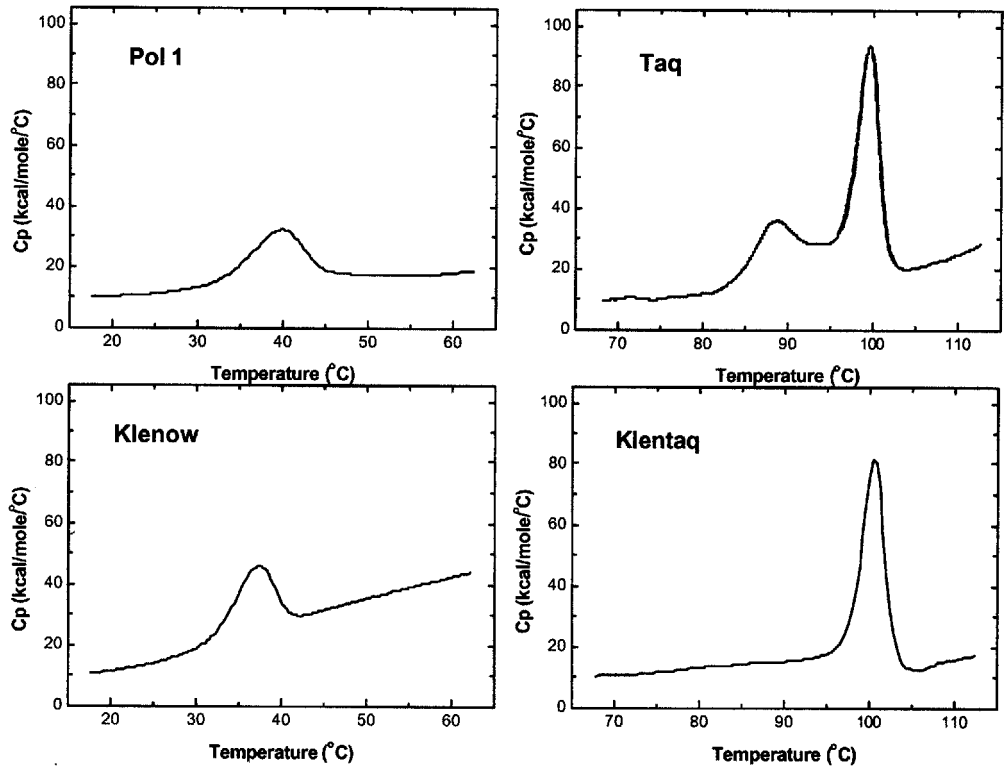

Figure 2 Characteristic heat-capacity curves for Pol 1, Klenow, *Taq* and Klentaq DNA polymerases, with buffer baselines subtracted and normalized for protein concentrations

All four proteins were denatured under identical buffer conditions in 10 mM K_2PO_4/10 mM EDTA/2 mM DTT (pH 9.5). Scans are shown with the same relative *y*-axis (C_p) and *x*-axis (°C) spacing.

wide variety of solution conditions were examined before finding identical conditions where all four proteins could be comparatively denatured directly without one or more of them precipitating (10 mM K_2PO_4, 10 mM EDTA and 2 mM DTT, pH 9.5). Further exploration of the denaturation of Klentaq and Klenow polymerases was also performed as a function of pH, scan rate and added methanol.

Each of the polymerases Klenow and Klentaq thermally denature as a single peak in DSC. Both the full-length polymerases, however, denature in a two-domain fashion. The excess heat-capacity curves for full-length Pol 1 and *Taq* can be deconvoluted into separate contributions from the 5′ nuclease and large fragment domains as shown in Figure 3 and discussed further below. The fitted values for the calorimetrically determined apparent thermodynamic parameters for the denaturation of the four proteins at pH 9.5 are shown in Table 1.

The four polymerases showed very limited reversibility upon rescanning, as commonly found for proteins of this size (*Taq* is 94 kDa, Pol 1 103 kDa, Klentaq 62 kDa and Klenow 68 kDa). Klenow, Pol 1 and the second peak of *Taq* are not reversible; isolated Klentaq is partially reversible (15–30%) and the 5′ nuclease peak for *Taq* is mostly reversible (85–100%). The problem of irreversibility in DSC is quite common [19,20], especially with proteins larger than 20 kDa and with thermophilic proteins. The total heat absorbed per mol of protein during calorimetric denaturation is a model-independent experimental value. However, because of the lack of full reversibility, the values in Table 1, with the exception of the 5′ nuclease peak of *Taq*, must be considered as apparent thermodynamic values. Despite their limitations, the calorimetric results reveal a number of interesting features of the structural stability of the polymerases.

Klenow and Klentaq denature calorimetrically with T_m values of 37 and 100 °C respectively under identical buffer conditions at pH 9.5 and temperature scan rates of 1 °C/min. The ΔH_{cal}^{app} for denaturation of Klentaq is approximately double that for Klenow at their respective T_m values. Since $d(\Delta H) = \Delta C_p \cdot dT$, where C_p is the heat capacity, the ΔH of any process with a positive ΔC_p (such as protein unfolding) will increase with temperature. Although the results of the present study do not allow determination of ΔC_p values for the polymerases, a relatively modest ΔC_p value of 1.8 kcal/mol per K (1 kcal ≡ 4.184 kJ) would put the ΔH_{cal}^{app} values for Klenow and Klentaq on the same line. Figure 2 also shows that Klentaq appears to unfold more co-operatively than Klenow (sharper peak), both as the isolated fragment and within the full-length protein.

When the full-length proteins are examined, the 5′ nuclease domains of both polymerases denature at lower temperatures than their corresponding large fragments. For *Taq* polymerase, the 5′ nuclease domain denatures as a separate peak with a T_m of approx. 89 °C. When Pol 1 is denatured, the peak for the 5′ nuclease domain overlaps with the Klenow domain and the two peaks must be resolved by non-linear regression [19], as shown in Figure 3.

Denaturations of Klentaq and Klenow were further examined as a function of pH, added methanol, scan rate and by optically monitored thermal denaturation. Figure 4 shows the relationship between T_m and pH for both Klentaq and Klenow. Only a limited pH range was obtained since pH values lower than those shown for each polymerase caused precipitation of the proteins during heating. Over the ranges examined, the T_m values decreased by −3.6 °C/pH unit for Klentaq and −4.8 °C/pH unit for Klenow. Values of pH < 9.5 caused precipitation of full-length *Taq* during

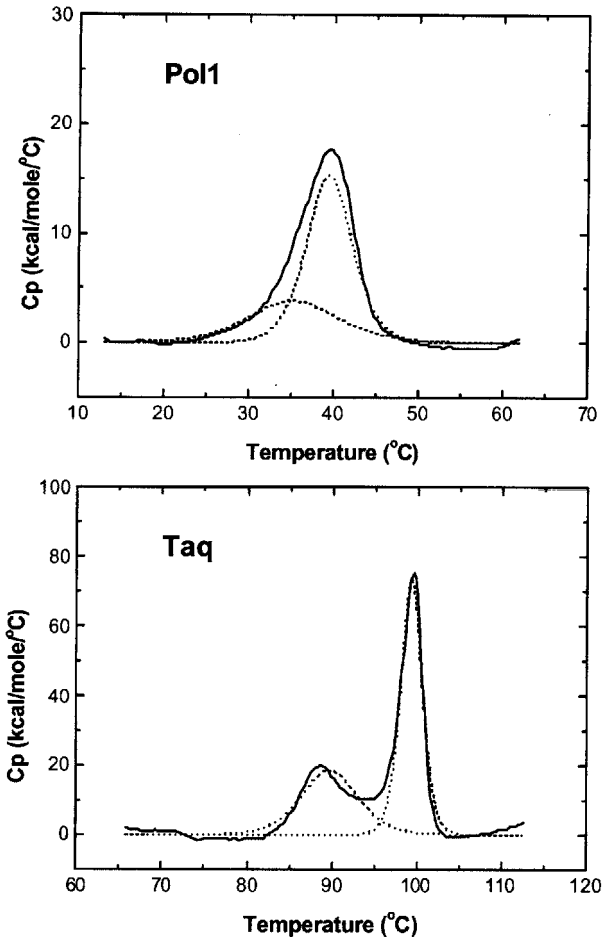

Figure 3 Fitted deconvolutions of multidomain denaturations of *Taq* and Pol 1 full-length polymerases

The dotted lines show the fits used to deconvolute the contributions of each melting domain in the full-length polymerases and to obtain the parameter values given in Table 1. Assignment of the first deconvoluted peak in Pol 1 to the 5′ nuclease domain is based on the unambiguous assignment of the first peak in the denaturation of *Taq* to the 5′ nuclease domain.

denaturation and, hence, the pH dependence of the full-length polymerases was not characterized.

Denaturations of Klentaq and Klenow were also performed in the presence of methanol concentrations between 0 and 10% (0–2.5 M). Low concentrations of methanol and other organic solvents are believed to decrease the contributions of the hydrophobic effect to protein stability [21,22]. The values of ΔH_{cal}^{app} for each polymerase remained constant within error (less than $\pm 10\%$) across all methanol concentrations (results not shown). T_m values for both Klentaq and Klenow decrease linearly as a function of added methanol, and are also shown in Figure 4. Klentaq is affected slightly more by the addition of methanol compared with Klenow, with values (slopes) of -2.6 and -1.8 °C/M (M, molar) respectively. It should be noted that, for studies on methanol dependence, the D424A ($Asp^{424} \rightarrow Ala$) mutant of Klenow was used, which is also known as KF exo− [23] (KF exo minus). KF exo− lacks the 3′ exonuclease or proofreading activity, and is the predominant form of Klenow used in functional studies. Whereas both variants were not characterized under all the conditions investigated in the present study, wild-type KF and KF exo− were found to behave identically under those conditions where both were examined (results not shown). All the Klenow

Table 1 Fitted parameters for the thermal denaturation of DNA polymerases at pH 9.5

DNA polymerase	T_m (°C) Peak 1†	T_m (°C) Peak 2†	T_m (°C) CD‡	ΔH_{cal} (kcal/mol)* Peak 1	ΔH_{cal} (kcal/mol)* Peak 2	ΔH_{vH} (kcal/mol)* CD‡
Taq§	88.9 ± 0.9	99.1 ± 0.1		147 ± 5	250 ± 4	
Klentaq§	–	100.2 ± 0.1	97.6 ± 0.1	–	244 ± 17	232 ± 14
Pol 1‖	36.7 ± 0.9	40.4 ± 0.9		56 ± 9	111 ± 2	
Klenow§	–	36.8 ± 0.5	35.0 ± 0.1	–	131 ± 2	110 ± 3

* Only peak 1 of *Taq* shows reversibility after rescanning under the conditions examined. Therefore the other thermodynamic quantities in this Table should formally be considered apparent thermodynamic values.

† Calorimetric results are reported for runs at the scan rate of 1 °C/min.

‡ For CD data, values are the means of fits to four different wavelengths as a function of temperature. As discussed in the text, T_m values for CD experiments are probably lower due to an effectively significantly slower scan rate in these experiments. T_m values for Klentaq and Klenow at the scan rate of 0.5 °C/min in DSC are 99.6 and 35.7 °C respectively.

§ *Taq*, Klentaq and Klenow excess heat capacity curves are fitted to a non-two-state model which treats each peak as a single transition but allows for asymmetry of the curve. T_m and ΔH_{cal} values were determined at the scan rate of 1 °C/min and are expressed as the means ± S.D. for three experiments.

‖ Pol 1 excess heat capacity curves are deconvoluted to two independent two-state transitions. Values are expressed as the means ± S.D. for three experiments.

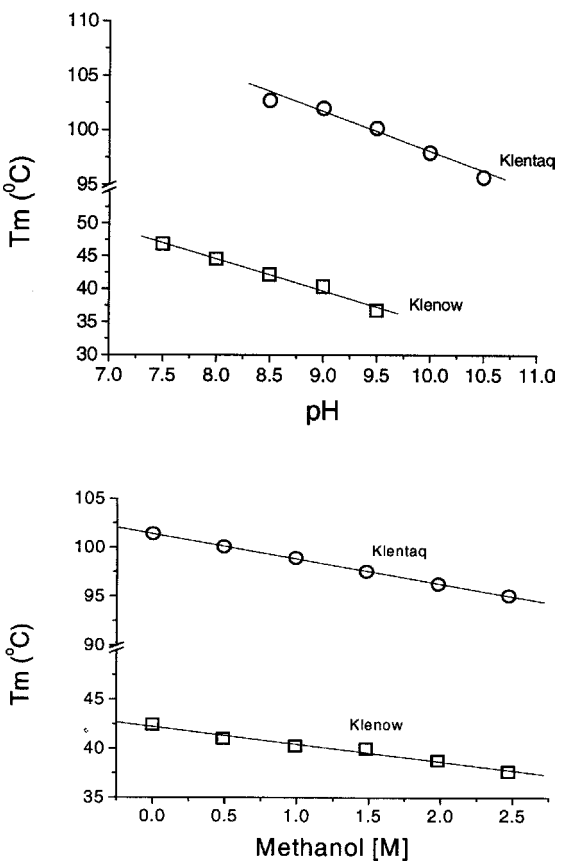

Figure 4 pH and methanol dependence of thermal denaturations of Klentaq and Klenow polymerases

The upper panel shows the T_m versus pH dependence for Klentaq and Klenow. T_m values at different concentrations of methanol are shown in the lower panel.

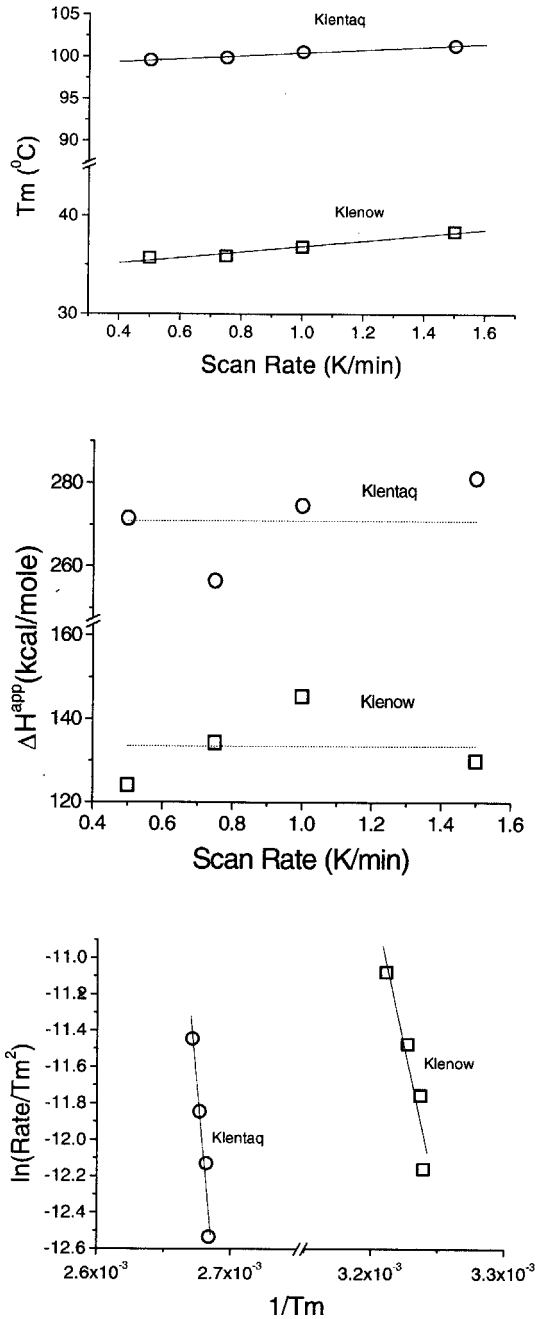

Figure 5 Scan rate dependence of thermal denaturations of Klentaq and Klenow polymerases

The top and middle panels show the T_m and ΔH_{cal}^{app} values obtained at different scan rates for the two polymerases. The dotted lines in the plot of ΔH_{cal}^{app} against scan rate show the mean ΔH_{cal}^{app} values for each polymerase. The bottom panel shows Arrhenius plots of the scan-rate-dependent changes in T_m as described in the text. The slopes of these lines provide the apparent activation energies of denaturation [17].

data presented in this paper are for wild-type Klenow except for the methanol data in Figure 4, which are for KF exo−.

Because the denaturations of the polymerases are irreversible, the calorimetric scan rate dependences for Klentaq and Klenow were also examined to assay for kinetic contributions during the thermal denaturation of the proteins. T_m values for both Klentaq and Klenow were found to be scan-rate-dependent, whereas ΔH_{cal}^{app} values were relatively scan-rate-independent. T_m and ΔH_{cal}^{app} values are shown in Figure 5 for both Klentaq and Klenow polymerases. Kinetic activation energies for irreversible denaturation were derived from the scan rate dependence of the DSC transitions using Arrhenius plots as described by Sanchez-Ruiz et al. [17], and these plots of ln(scan rate/T_m^2) against $1/T_m$ are also given in Figure 5. The slopes of these plots provide the activation energies for irreversible denaturation, which are 157 and 67 kcal/mol for Klentaq and Klenow respectively.

Thermal denaturations of Klenow and Klentaq were also monitored optically using CD spectrometry. The CD spectra at room temperature for both proteins reflect the high α-helical structure of the two proteins (Figure 6). As heat denatures the proteins, their secondary structure is disrupted, and the transition region of the denaturation profile can be analysed using a van't Hoff approach (Figure 6). Simultaneous fitting of the native and denatured baselines along with fitting of the transition region to the van't Hoff equation yields the van't Hoff enthalpy of unfolding, ΔH_{vH}, and the T_m of unfolding for the protein, as described in the Experimental section, and these values are presented in Table 1. The van't Hoff enthalpies ΔH_{vH} determined from the CD monitored denaturations were essentially identical with the ΔH_{cal} values for Klenow and Klentaq. This agreement suggests that the unfolding process is very nearly two-state, as agreement between calorimetric and van't Hoff enthalpies is one of the defining characteristics of a two-state denaturation process [19,20,29]. Even though $\Delta H_{vH} = \Delta H_{cal}$, the CD data were also found to be irreversible, further implying the population of an irreversible state after equilibrium denaturation (see the Discussion section). It is straightforward to understand the lower T_m values obtained from the CD titrations as scan rate dependence effects. The calorimetric T_m values presented in Table 1 are from scans performed at 1 °C/min, as this is the most commonly used and reported scan rate in DSC experiments. The CD titrations are necessarily performed, as described in the Experimental section, by heating to each new temperature and allowing a 5 min equilibration time at that temperature; slower scan rates, as shown in Figure 5, will yield lower T_m values.

It is noteworthy that the $\Delta\varepsilon$ baselines for the denatured states (upper baselines) of both proteins still exhibit negative CD intensity in the 220 nm wavelength range. Although models of random-coil conformation polypeptides are classically known to have positive CD intensity in this wavelength range, as depicted in any textbook introduction to CD, actual denatured proteins almost universally do not exhibit positive CD intensities in this region of their spectra (e.g. see [24,25]). This has often been attributed to the presence of a residual structure of the denatured state of the protein. The $\Delta\varepsilon$ versus temperature profiles for Klenow and Klentaq shown in Figure 6 are characteristic examples of such denaturations.

DISCUSSION

Two-domain melting of the polymerases

Although both *Taq* and Pol 1 polymerases contain three structural/functional domains [2,3,26], they do not denature in three peaks. In DSC, Klenow and Klentaq both denature in a single peak. Correspondence between the calorimetric and van't Hoff ΔH values (Table 1) indicates that thermal transition is effectively two-state for both Klenow and Klentaq. The melting transition for Klentaq in DSC, however, is actually slightly more co-operative (sharper peak) than a two-state transition, possibly due to the onset of a kinetically controlled irreversible step as discussed below in the Scan rate dependence subsection. The single peak, two-state behaviour of Klenow and Klentaq indicates a tight

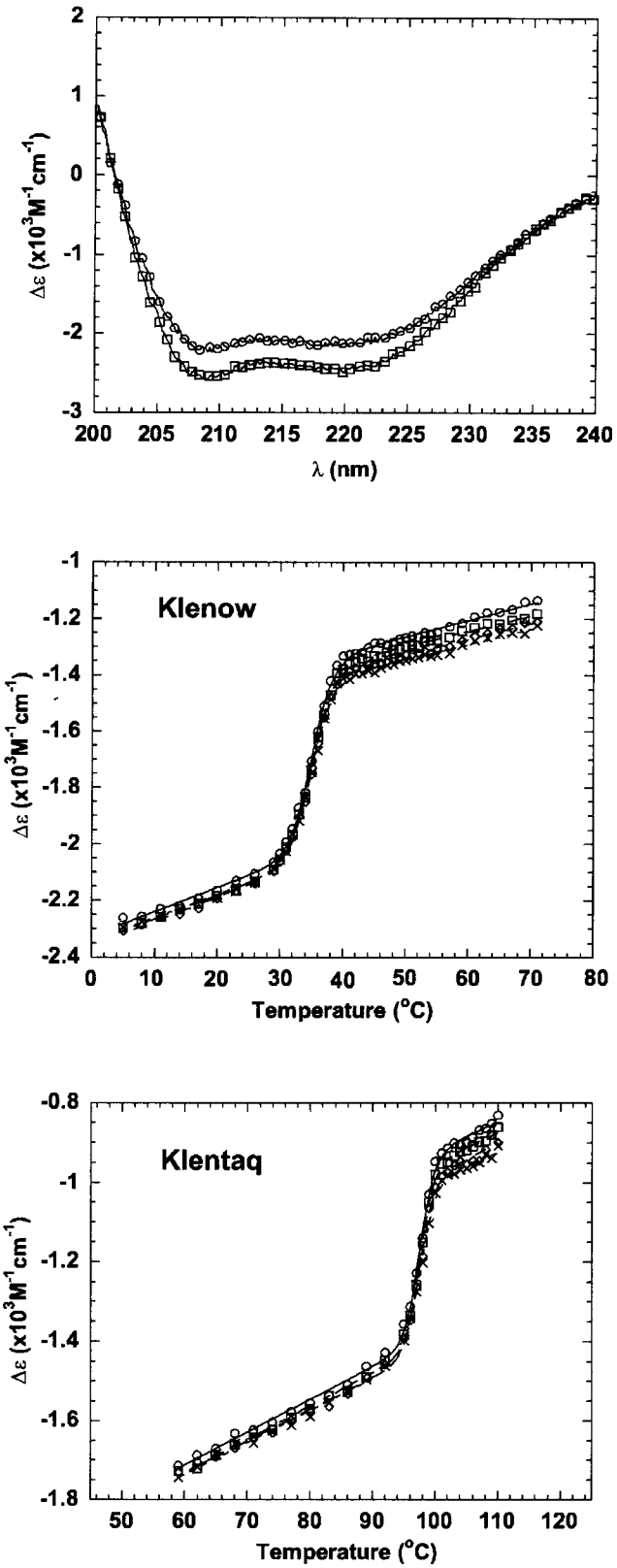

Figure 6 Thermal denaturations of Klenow and Klentaq monitored by CD

The top panel shows the room temperature CD spectra of the two proteins. The upper spectrum (○) corresponds to Klenow and the lower spectrum (□) to Klentaq. The bottom two panels show the thermal denaturations of the two proteins monitored at four different wavelengths (in both plots, data at 219, 220, 221 and 222 nm are denoted by ×, ◇, □ and ○ respectively). Lines show the fits to a modified van't Hoff equation as described in the text.

folding/unfolding coupling between the polymerase and the 3′ exonuclease functional domains within the large fragments. In contrast, full-length *Taq* polymerase denatures in two cleanly separated transitions, with the 5′ exonuclease domain denaturing approx. 10 °C before the Klentaq domain. Full-length *E. coli* Pol 1 denatures as a single peak, but the one that easily deconvolutes into two separate transitions. Based on the denaturation behaviour of full-length *Taq*, it is straightforward to assign the two melting domains in Pol 1 to the 5′ nuclease domain and the Klenow fragment.

The melting data show that the 5′ nuclease domain of *Taq* will in fact denature during the normal course of PCR, where the temperatures used for melting the DNA duplexes formed during each cycle are typically 94–97 °C. The 5′ nuclease domain of *Taq* is the only reversibly denaturing peak in DSC; thus, this domain will denature and refold during each cycle of a typical PCR run using full-length *Taq*. This previously unknown characteristic of the 5′ nuclease domain has been particularly fortuitous for the development of real-time PCR assays, many of which require the 5′ nuclease activity of the protein.

Inter-domain interactions

A comparison of the T_m values for the full-length proteins versus the Klentaq and Klenow fragments suggests that the unfolding domains interact in both proteins, but in opposite ways. When the 5′ nuclease domain is removed from full-length *Taq*, the T_m value for the Klentaq domain increases by approx. 1 °C. Thus the interaction between the Klentaq and 5′ nuclease domains in full-length *Taq* is slightly unfavourable for the Klentaq domain, and the Klentaq fragment is slightly more stable on its own. This observation is in agreement with an increase in the time of stability at 97.5 °C found for Klentaq versus *Taq* when measured using the traditional loss of activity assay [27]. Conversely, removal of the 5′ nuclease domain from Pol 1 leads to an apparent 3.6 °C T_m destabilization of the Klenow domain. Thus the presence of the 5′ nuclease domain in Pol 1 appreciably stabilizes the rest of the protein.

T_m versus growth temperature

Another interesting feature of our results is that Pol 1 and Klenow begin denaturing at or slightly above 37 °C, the optimal growth temperature of *E. coli*, whereas *Taq*/Klentaq polymerase is stable even at much higher temperatures than the 70–72 °C optimal growth range [1,28] of *T. aquaticus*. The melting temperatures near 37 °C for Klenow and Pol 1 indicate that additional factors such as ion binding, molecular crowding, DNA and/or nucleotide binding and possibly natural osmolytes must be acting *in vivo* to keep Pol 1 from denaturing at the optimal growth temperature of *E. coli*. Thus, unlike *Taq* polymerase, the intrinsic thermal stability of *E. coli* Pol 1 polymerase is not adequate or only marginally adequate to preserve its native state *in vivo*.

pH dependence

The dependence of T_m on pH is similar for both Klentaq and Klenow, both showing a modest decrease in T_m with increasing pH. The near linearity and lack of a large excursion of T_m versus pH indicate that there are no stability-linked amino acid residues with pK_a values in this range. However, deprotonation is destabilizing for both proteins. Normally, the pH-dependent shift in T_m and ΔH for a protein can be used to determine the ΔC_p for

denaturation of that protein [29]. However, the irreversible nature of these transitions (and their scan rate dependence, see below) implies that such an analysis would not be thermodynamically valid for these data, and could be misleading.

Interestingly, almost all commercially available reaction buffers used for PCR are Tris at or near pH 9 (measured at room temperature). Tris buffer has one of the largest temperature coefficients of any commonly used biological buffer, and will decrease in pH by 1–2 pH units as the temperature is increased during PCR cycling. Therefore, fortuitously, the use of Tris as the buffer of choice for PCR means that, during each high temperature portion of a normal PCR cycle, the polymerase will be additionally protected by the concomitant decrease in pH of the reaction buffer. The calorimetric experiments described in the present study were all performed in phosphate buffer, which has almost no temperature dependence. Calorimetrically, we found that, although lowering the pH from 10.5 to 8.5 increases the T_m, at pH values lower than 8.5 Klentaq precipitates on full denaturation. This raises the question of why *Taq*/Klentaq does not precipitate during PCR. First, full calorimetric denaturation of *Taq*/Klentaq involves heating the protein to 110–115 °C, temperatures far above those used in PCR. Furthermore, at lower pH values, the shift in T_m to higher temperatures also means that the beginning of the melting transition is shifted to higher temperatures. The high temperature DNA dissociation step in PCR is generally performed at 94–97 °C. At pH 9.5, Klentaq has just started to melt by this temperature (see Figure 2), whereas at pH 8.5 its melting transition does not begin until above 98 °C.

Methanol dependence

The effect of low concentrations of methanol on proteins is generally attributed to the increased hydrophobicity of the solvent, which decreases the favourability of the burial of hydrophobic regions of the protein in the native state (e.g. see [21,22]). Changes in T_m with the addition of methanol for Klentaq and Klenow (-2.6 and -1.8 °C/M respectively) are similar to the methanol dependence observed for other proteins, including lysozyme [21], lipase [30] and cytochrome *c* [22].

Because $d(\Delta H) = \Delta C_p \cdot dT$, by definition, the denaturation enthalpy will increase with temperature. Since the denaturation T_m and ΔH of a protein can be shifted with pH [29], an effective plot of ΔH against T_m can be constructed and extrapolated to temperatures far beyond those under which the protein is stable [22,29,31]. Convergence of extrapolated normalized denaturation enthalpies has been found to occur close to 100 °C for basically all proteins examined to date [22,29,31]. Different studies of the so-called enthalpy and entropy convergence temperatures in protein folding have led to the conclusion that, at these temperatures (which are 100 °C for ΔH and approx. 112 °C for ΔS), the hydrophobic contributions to ΔH and ΔS are zero (e.g. [22,31]). Klentaq denatures exactly at the ΔH convergence temperature, whereas Klenow denatures far below it; therefore, these models might suggest that the balance of hydrophilic versus hydrophobic stabilizing forces in the two proteins might be quite different. However, methanol affects both proteins similarly and, in fact, has a larger effect on Klentaq when compared with Klenow. Thus Klentaq seems to be quite well stabilized by hydrophobic effects, perhaps more so when compared with Klenow, even at the enthalpy convergence temperature. These results might suggest that the hydrophobic contributions to ΔH are not zero for Klentaq at the enthalpy convergence temperature, or that there is a very strong favourable hydrophobic entropy effect for Klentaq at 100 °C.

Scan rate dependence

Thermal denaturations of both Klentaq and Klenow were found to be dependent on the rate of heating in the calorimeter. As discussed above in the Results section, the T_m data obtained by thermal denaturation monitored by CD also indicate a scan rate effect. Scan rate dependence is generally regarded as being indicative of at least partial kinetic control of the melting process. The Lumry–Eyring reaction scheme is the most common framework used for modelling irreversible denaturation [32]. In this model, a native protein (N) undergoes two transitions: an equilibrium transition to an unfolded state (U), followed by a kinetic transition to an irreversibly denatured state (I):

$$N \overset{K_{eq}}{\longleftrightarrow} U \overset{k_3}{\longrightarrow} I$$

Support for this model for Klentaq and Klenow comes from an agreement between the calorimetric unfolding enthalpies and van't Hoff enthalpies determined from CD denaturations, as this is the primary distinguishing characteristic of a two-state denaturation process ($N \leftrightarrow U$) [19,20,29], whereas the irreversibility of thermal melting both in the calorimeter and the spectrophotometer and also the scan rate dependence in DSC indicate a subsequent $U \rightarrow I$ kinetic step.

Sanchez-Ruiz and co-workers [17] and other authors [35,36] have developed a number of different methods of obtaining Arrhenius plots from the scan rate dependence of irreversible denaturations. Most of these approaches begin by assuming that the rate $U \rightarrow I$ is higher than the back rate $U \rightarrow N$ and thus analyse the Arrhenius behaviour of the scan rate dependence as a single-step $N \rightarrow I$ process. Arrhenius analysis of the scan rate dependence then provides an activation energy for the full irreversible denaturation of the protein $N \rightarrow I$. If either the $N \rightarrow U$ step or the $U \rightarrow I$ step is rate-limiting, then the obtained activation energy would be most correlated with this step, but definitive determination of the rate-limiting step is difficult. Since Sanchez-Ruiz and co-workers [17,33,34] and other authors [35,36] report more or less equivalent activation energies with each of the different Arrhenius-based analysis methods, we have demonstrated only one of these methods here (in Figure 5).

Although only a small number of proteins have been analysed by these methods, the activation energy for the irreversible denaturation of Klenow (67 kcal/mol) is similar to the majority of the proteins examined previously, whereas the E_a for Klentaq (157 kcal/mol) is not the highest but is one of the highest E_a values reported so far (see [36] for a summary). Within the formalism of the model, the higher activation barrier for Klentaq indicates a slower irreversible step, and implies that, in some cases, Klentaq unfolds to a large extent (i.e. an $N \leftrightarrow U$ equilibrium may be largely established) before irreversible denaturation begins [36]. Extension of similar analyses for other proteins has suggested that, in some (few) situations where the kinetic barrier to irreversible denaturation is large and its rate is low, the enthalpies derived from completely irreversible DSC curves may in fact be considered equilibrium thermodynamic parameters [36–38]. However, establishment of a widely accepted set of criteria for determining when such equivalences can be made and when they cannot is still an area of active research [38,39].

The significantly higher activation barrier for irreversible denaturation of Klentaq versus Klenow does indicate that Klentaq is significantly kinetically stabilized when compared with Klenow. In other words, temperatures at which each of them would denature (i.e. within the ranges of each of their DSC transition curves), a higher E_a for Klentaq indicates it will take longer to irreversibly denature than Klenow. Again, since these

kinetic analyses are actually reflective of the entire process N → I, the slower process in Klentaq may be either the N → U step or the U → I step. This analysis does mean, however, that *in vitro* as well as *in vivo* Klentaq/*Taq* would be protected from irreversible damage at temperatures near and above its T_m for longer than Klenow at temperatures near and above its T_m.

Concluding comments

Even though calorimetric denaturations of *Taq* and *E. coli* Pol 1 polymerases are irreversible and preclude a strict thermodynamic analysis, a parallel examination of the melting behaviours of the polymerases and their large fragments has revealed a number of similarities and differences between the two proteins. Both of the full-length polymerases melt in a two-domain fashion, with the 5′ nuclease domain melting separately and first. The influence of the 5′ nuclease domain on the rest of the protein is opposite in the two proteins: it is stabilizing in *E. coli* Pol 1 and destabilizing in *Taq*. The pH and methanol dependence of the melting temperatures for Klentaq and Klenow are very similar (even though under any parallel solution condition their absolute values are separated by more than 60 °C), suggesting little or no difference in the contributions of hydrophobic forces and protonation/deprotonation effects to the stability of the two species of polymerase. Finally, Arrhenius analysis of the scan rate dependence for denaturation of Klentaq and Klenow indicates a substantially greater kinetic barrier to irreversible denaturation of Klentaq when compared with Klenow.

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REFERENCES

1 Perler, F. B., Kumar, S. and Kong, H. (1996) Thermostable DNA polymerases. Adv. Protein Chem. **48**, 377–435
2 Lawyer, F. C., Stoffel, S., Saiki, R. K., Myambo, K., Drummond, R. and Gelfand, D. H. (1989) Isolation, characterization, and expression in *Escherichia coli* of the DNA polymerase gene from *Thermus aquaticus*. J. Biol. Chem. **264**, 6427–6437
3 Kim, Y., Eom, S. H., Wang, J., Lee, D.-S., Suh, S. W. and Steitz, T. A. (1995) Crystal structure of *Thermus aquaticus* DNA polymerase. Nature (London) **376**, 612–616
4 Korolev, S., Nayal, M., Barnes, W. M., DiCera, E. and Waksman, G. (1995) Crystal structure of the large fragment of *Thermus aquaticus* DNA polymerase I at 2.5-Å resolution: structural basis for thermostability. Proc. Natl. Acad. Sci. U.S.A. **92**, 9264–9268
5 Eom, S. H., Wang, J. and Steitz, T. A. (1996) Structure of *Taq* polymerase with DNA at the polymerase active site. Nature (London) **382**, 278–281
6 Ollis, D. L., Brick, P., Hamlin, R., Xuong, N. G. and Steitz, T. A. (1992) Structure of large fragment of *Escherichia coli* DNA polymerase I complexed with dTMP. Nature (London) **313**, 762–766
7 Barnes, W. M. (1992) The fidelity of *Taq* polymerase catalyzing PCR is improved by an N-terminal deletion. Gene **112**, 29–35
8 Szilagyi, A. and Zavodszky, P. (2000) Structural differences between mesophilic, moderately thermophilic and extremely thermophilic protein subunits: results of a comprehensive survey. Structure (London) **8**, 493–504
9 Petsko, G. A. (2001) Structural basis of thermostability in hyperthermophilic proteins, or 'there's more than one way to skin a cat'. Methods Enzymol. **334**, 469–478
10 Sterner, R. and Liebl, W. (2001) Thermophilic adaptation of proteins. Crit. Rev. Biochem. Mol. Biol. **36**, 39–106
11 Rees, D. C. and Robertson, A. D. (2001) Some thermodynamic implications for the thermostability of proteins. Protein Sci. **10**, 1187–1194
12 Jaenicke, R. and Bohm, G. (1998) The stability of proteins in extreme environments. Curr. Opin. Struct. Biol. **8**, 738–748
13 Rees, D. C. and Adams, M. W. (1995) Hyperthermophiles: taking the heat and loving it. Structure **3**, 251–254
14 Minkley, Jr, E. G., Leney, A. T., Bodner, J. B., Panicker, M. M. and Brown, W. E. (1984) *Escherichia coli* DNA polymerase I. Construction of a polA plasmid for amplification and an improved purification scheme. J. Biol. Chem. **259**, 10386–10392
15 Datta, K. and LiCata, V. J. (2003) Salt dependence of DNA binding by *Thermus aquaticus* and *Escherichia coli* DNA polymerases. J. Biol. Chem. **278**, 5694–5701
16 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. **72**, 248–254
17 Sanchez-Ruiz, J. M., Lopez-Lacomba, J. L., Cortijo, M. and Mateo, P. L. (1988) Differential scanning calorimetry of the irreversible thermal denaturation of thermolysin. Biochemistry **27**, 1648–1652
18 Eftink, M. R. and Ramsay, G. D. (1994) Methods Enzymol. **240**, 615–645
19 Sturtevant, J. M. (1987) Biochemical applications of differential scanning calorimetry. Annu. Rev. Phys. Chem. **38**, 463–488
20 Privalov, P. L. (1982) Stability of proteins. Proteins which do not present a single cooperative system. Adv. Protein Chem. **35**, 1–104
21 Velicelebi, G. and Sturtevant, J. M. (1979) Thermodynamics of the denaturation of lysozyme in alcohol–water mixtures. Biochemistry **18**, 1180–1186
22 Fu, L. and Freire, E. (1992) On the origin of the enthalpy and entropy convergence temperatures in protein folding. Proc. Natl. Acad. Sci., U.S.A. **89**, 9335–9338
23 Derbyshire, V., Freemont, P. S., Sanderson, M. R., Beese, L., Friedman, J. M., Joyce, C. M. and Steitz, T. A. (1988) Genetic and crystallographic studies of the 3′,5′-exonucleolytic site of DNA polymerase I. Science **240**, 199–201
24 Johnson, W. C. (1988) Secondary structure of proteins through circular dichroism spectroscopy. Annu. Rev. Biophys. Biophys. Chem. **17**, 145–156
25 Sreerama, N., Venyaminov, S. Y. and Woody, R. W. (2000) Estimation of protein secondary structure from circular dichroism spectra: inclusion of denatured proteins with native proteins in the analysis. Anal. Biochem. **287**, 243–251
26 Joyce, C. M. and Steitz, T. A. (1994) Function and structure relationships in DNA-polymerases. Annu. Rev. Biochem. **63**, 777–822
27 Barnes, W. M. (1995) Thermostable DNA polymerase with enhanced thermostability and enhanced length and efficiency of primer extension. U.S. Pat. 5,436,149
28 Brock, T. D. (1974) In Bergey's Manual of Determinative Bacteriology (Buchanan, R. E. and Gibbons, N. E., eds.), 8th edn, p. 285, Williams and Wilkins, Baltimore, MD
29 Privalov, P. L. and Khechinashvili, N. N. (1974) A thermodynamic approach to the problem of stabilization of globular protein structure: a calorimetric study. J. Mol. Biol. **86**, 665–684
30 Tanaka, A. (1998) Differential scanning calorimetric studies on the thermal unfolding of *Pseudomonas cepacia* lipase in the absence and presence of alcohols. J. Biochem. (Tokyo) **123**, 289–293
31 Murphy, K. P. and Gill, S. J. (1990) Group additivity thermodynamics for dissolution of solid cyclic dipeptides into water. Thermochim. Acta **172**, 11–20
32 Lumry, R. and Eyring, H. (1954) Conformational changes of proteins. J. Phys. Chem. **58**, 110–120
33 Galisteo, M. L., Conejero-Lara, F., Nunez, J., Sanchez-Ruiz, J. M. and Mateo, P. L. (1992) A calorimetric approach to the kinetics of irreversible thermal denaturation of proteins. Thermochim. Acta **199**, 147–157
34 Galisteo, M. L., Mateo, P. L. and Sanchez-Ruiz, J. M. (1991) Kinetic study on the irreversible thermal denaturation of yeast phosphoglycerate kinase. Biochemistry **30**, 2061–2066
35 Lepcock, J. R., Ritchie, K. P., Kolios, M. C., Rodahl, A. M., Heinz, K. A. and Kruuv, J. (1992) Influence of transition rates and scan rate on kinetic simulations of differential scanning calorimetry profiles of reversible and irreversible protein denaturation. Biochemistry **31**, 12706–12712
36 Vogl, T., Jatzke, C., Hinz, H.-J., Benz, J. and Huber, R. (1997) Thermodynamic stability of annexin V E17G: equilibrium parameters from an irreversible unfolding reaction. Biochemistry **36**, 1657–1668
37 Freire, E., van Osdol, W. W., Mayorga, O. L. and Sanchez-Ruiz, J. M. (1990) Calorimetrically determined dynamics of complex unfolding transitions in proteins. Annu. Rev. Biophys. Biophys. Chem. **19**, 159–188
38 Sanchez-Ruiz, J. M. (1992) Theoretical analysis of Lumry–Eyring models in differential scanning calorimetry. Biophys. J. **61**, 921–935
39 Luybarev, A. E. and Kurganov, B. I. (2000) Analysis of DSC data relating to proteins undergoing irreversible thermal denaturation. J. Therm. Anal. Calorim. **62**, 51–62

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